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CERTIFICATE OF TRANSLATION

I, SHUSAKU YAMAMOTO, patent attorney of Fifteenth Floor, Crystal Tower, 1-2-27 Shiromi, Chuo-ku, Osaka 540-6015, Japan HEREBY CERTIFY that I am acquainted with the English and Japanese languages and that the attached English translation is a true English translation of what it purports to be, a translation of Japanese Patent Application No. 2000-25596 filed on 2 February, 2000 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION.

Additionally, I verify under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Executed this 12th day of February, 2003.

SHUSAKU YAMAMOTO

(Translation)

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[Title of the Invention]	GENE INTRODUCTION METHOD AND GENE INTRODUCTION VECTOR
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[List of the Documents]

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[Item]	Abstract	1

[Proof] Required

(Translation)

[Name of the Document] SPECIFICATION

[Title of the Invention] Gene Introduction Method and Gene Introduction Vector

[Claims]

[Claim 1] A method for introducing an exogenous gene, characterized by repeatedly freezing and thawing a mixture solution of the exogenous gene and an inactivated Sendai virus and thereafter fusing the virus, in which the exogenous gene is encapsulated, with a cell to introduce the exogenous gene into the cell.

[Claim 2] A gene introduction vector, wherein the vector is an inactivated Sendai virus in which an exogenous gene is encapsulated.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a gene introduction method and a gene introduction vector. More particularly, the present invention relates to a method of easily and reliably introducing an exogenous gene into an animal cell, and a gene introduction vector for use in this method, which can be cryopreserved for a long time.

[0002]

[Prior Art]

An exogenous gene is introduced into a cell or a fertilized egg for the purpose of functional analysis of a gene, expression of a large amount of a useful protein,

production of a transgenic animal, gene therapy, and the like. Examples of known gene introduction methods include a calcium phosphate method, a lipofection method using liposome, a method using erythrocyte ghosts, an electroporation method, a method using a retrovirus or an adenovirus as a vector, and a method of injecting a small amount of genes into a cell using a glass pipette, which are used according to the type of a cell, the purpose, or the like.

[0003]

The present inventors have utilized the fusion capability of Sendai virus (hemagglutinating virus of Japan: hereinafter referred to as HVJ) to develop an ultraviolet ray inactivated HVJ-liposome (J. Biol. Chem. 266(6):3361-3364, 1991), and have conducted introduction of a gene into a cultured cell or in vivo tissue, a gene therapy experiment, and the like, and reported its usefulness (e.g., see Proc. Natl. Acad. Sci. USA 93:11421-11425, 1996). Moreover, a method of using the HVJ-liposome as a gene introduction vector was confirmed to have a very low level of cytotoxicity according to a study of safety using a monkey, and is expected to be applied to gene therapy for a human.

[0004]

[Problems to be Solved by the Invention]

However, the HVJ-liposome as a gene introduction vector has problems, such as, it cannot be preserved for a long time and has to be prepared upon use, a skill is required for preparation of the liposome, and the like.

[0005]

The present invention is provided to solve the above-described problems. An object of the present invention is to provide a gene introduction method with ease of use and excellent gene introduction efficiency, where the excellent properties of the inactivated HVJ are utilized.

[0006]

Another object of the present invention is to provide a gene introduction vector which can be preserved for a long time.

[0007]

[Means for Solving the Problems]

The present application provides, as an invention solving the above-described problems, a method for introducing an exogenous gene characterized by repeatedly freezing and thawing a mixture solution of the exogenous gene and an inactivated Sendai virus and thereafter fusing the virus, in which the exogenous gene is encapsulated, with a cell to introduce the exogenous gene into the cell.

[0008]

Further, the present application provides a gene introduction vector, which is an inactivated Sendai virus in which an exogenous gene is encapsulated.

Hereinafter, embodiments of the present invention will be described in detail.

[0009]

[Embodiments of the Invention]

An inactivated HVJ for use in the present invention may be obtained by inactivating an HVJ using ultraviolet

light or the like in accordance with a method described in a publication (J. Biol. Chem. 266(6):3361-3364, 1991).

[0010]

The exogenous gene is not particularly limited. A genomic gene, its cDNA, or the like can be used as the exogenous gene. The gene DNA or cDNA can be incorporated into an animal cell expression vector having, for example, a promoter/enhancer sequence, a splicing region, a poly(A) addition site, and the like, and then encapsulated in the inactivated HVJ virus.

[0011]

An exogenous gene can be encapsulated in an inactivated HVJ virus by mixing the virus with the gene DNA in a buffer. The buffer is not particularly limited. TE, PBS, BSS, or the like can be appropriately used as the buffer. Particularly, TE is preferable.

[0012]

As a characteristic feature of the present invention, the mixture of the inactivated HVJ virus/the exogenous virus is repeatedly frozen and thawed. The repetition of the freezing and thawing promotes the transfer of the exogenous gene into the HVJ virus. The number of times of the freezing and thawing can be appropriately determined depending on the size or type of the exogenous gene, the type of a cell into which the recombinant HVJ virus transfected, and is at least 2, preferably at least 5, and even more preferably about 15 to about 20. The freezing can be performed using a refrigerator, dry ice, or the like. The thawing can be performed by thawing a cell at room temperature or the like. The freezing time or the thawing

time is not particularly limited and can be appropriately determined.

[0013]

The thus-prepared recombinant HVJ virus can be preserved in a frozen state for a long time (at least 3 months). The recombinant HVJ virus can be sealed in a frozen state, stored, and transported, for example.

[0014]

When gene introduction is performed using the recombinant HVJ virus, frozen virus is thawed and then is transfected into a host cell. In this case, as a procedure for the transfection, for example, a method of adding a solution of the recombinant HVJ virus to a medium of cultured cells, or the like can be adopted. The transfection reaction is performed for at least 5 minutes and no more than 10 hours when the reaction temperature is 37°C. When the reaction is performed beyond 10 hours, cells are fused to generate coenocytes. Therefore, the reaction time of about 20 to 30 minutes is typically preferably adopted, although the reaction time varies depending on the cell type.

[0015]

With the above-described method, the recombinant virus is fused with the cultured cell, whereby the exogenous gene encapsulated in the virus is expressed in the host cell.

Hereinafter, the method of the present invention will be more specifically and concretely described by way of examples. The present invention is not limited to examples below.

[0016]

[Examples]

Example 1

The luciferase gene was used as an exogenous gene. After freezing and thawing a recombinant HVJ virus various times, the gene was introduced into cultured cells.

[0017]

To 500 µl of TE, 750 µg of luciferase expression vector pcOriPLuc and various concentrations of HVJ virus were mixed. The HVJ virus concentration was adjusted to 10, 25, 50, or 100 HAU/il. This solution was divided into twelve aliquots, each of which was preserved at 4°C with dry ice, and frozen and thereafter thawed; this was repeated up to thirty times. A solution which had experienced a predetermined number of times of freezing and thawing was added to a medium for BHK-21 cells (24 well-dish, 4×10^4 cells/dish, 0.5 ml DMEM, 10% FCS). After the solution was allowed to react with 5% CO_2 at 37°C for 20 minutes, the solution was washed with PBS, and another 0.5 ml of the culture solution was added and cultured for 24 hours.

[0018]

The medium was removed. After 500 µl of 1×Cell Culture Lysis Reagent (Promega) was added to the cells to dissolve the cells, the solution was placed in a microtube so as to be centrifuged. From 20 µl of the resultant supernatant, the luciferase activity was measured by using Promega Luciferase Assay System and Lumat LB9501 Luminophotometer. The measurements were taken three times for each solution, and a mean value was obtained.

[0019]

The results are shown in Figure 1. The luciferase activity increased with an increase in the number of times of freezing and thawing for recombinant HVJ virus. With twenty times of freezing and thawing, tenfold or more luciferase expression was observed as compared to that observed with three times of freezing and thawing. From these results, it was confirmed that, under the conditions used in this example, the number of times of freezing and thawing for the recombinant HVJ virus is preferably five or more, and more preferably about 15 to about 20.

Example 2

After freezing and thawing a recombinant HVJ virus similar to that used in Example 1 thirty times, gene introduction efficiency into the cell was examined while ensuring that the same number of viruses were added to the host cell.

[0020]

The results are shown in Figure 2. In Figure 2, at 500 HAU on the X axis, for example, the solution having a virus concentration of 10 HAU/il was added in an amount of 50 μl, as opposed to 5 μl for the 100 HAU/μl solution. As shown in Figure 2, the gene expression efficiency of the solution having a virus concentration of 100 HAU/il decreased by about 50% as compared to that associated with a concentration of 10 to 50 HAU/il. From these results, it was confirmed that, under the conditions in this example, the recombinant virus concentration was preferably in a range of 10 to 50 HAU/il.

[0021]

Moreover, after freezing and thawing a recombinant HVJ virus twenty-nine times, freezing was performed for a thirtieth time, and the solution was preserved in this frozen state for a week, and thereafter thawed to be added to the cells. As a result, the recombinant HVJ virus which was preserved in a frozen state for one week also exhibited the same level of luciferase gene expression as that of the virus which experienced thirty consecutive times of freezing and thawing.

Example 3

Recombinant HVJ viruses were prepared by employing various amounts of luciferase expression vector, and the gene introduction efficiency into a host cell was examined.

[0022]

The amount of HVJ virus was 50 HAU per μl of TE. The amount of luciferase expression vector pcOriPLuc was 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, or 5.5 μg per μl of TE. Twenty times of freezing and thawing were carried out, with a final solution amount of 100 μl, and thereafter luciferase activity was measured by the same method as that of Example 1.

[0023]

The results are shown in Figure **3**. The expression amount increased in a dose-dependent manner until the added amount of expression vector pcOriPLuc (about 9.5 kb) as an exogenous gene reached 1.5 μg; thereafter, there was hardly any change in the expression amount. From the above results, it was confirmed that, under the conditions employed in this example, it is preferable to cause

1.5 μg/μl or more of exogenous gene DNA to be expressed.

Example 4

The gene introduction efficiency into the host cell was examined while varying the types of buffers used for the preparation of a recombinant HVJ virus vector.

[0024]

The amount of HVJ virus was 50 HAU per 1l of buffer, and the amount of luciferase expression vector pcOriPLuc was 1.5 μg/μl. As the buffer, TE, PBS, or BSS, or those obtained by adding saccharose to these buffers at a final concentration of 0 mM, 20 mM, 40 mM, or 60 mM were used. Twenty times of freezing and thawing were carried out, with a final solution amount of 100 μl, and thereafter luciferase activity was measured by the same method as that of Example 1.

[0025]

The results are shown in Figure 4, it was confirmed that, under the conditions employed in this example, it is preferable to use TE alone as the buffer for the preparation of a recombinant HVJ virus.

Example 5

Gene introduction employing inactivated HVJ-liposome (of the AVE type having the most excellent gene introduction efficiency, which is a conventional gene introduction vector, and the method according to the present invention were compared.

[0026]

The amount of HVJ-liposome or HVJ virus was 50 HAU per

μl of TE, and the amount of luciferase expression vector pcOriPLuc was 1.5 μg/μl. The number of times of freezing and thawing for the recombinant HVJ virus was twice or fifteen times. The other conditions were the same as in Example 1, except that human fetus kidney cell line HEK293 was used as host cells.

[0027]

The results are shown in Figure 5. It was confirmed that the method according to the present invention which repeats fifteen times of freezing and thawing of the recombinant HVJ virus is far more excellent in gene introduction efficiency than the conventional method employing HVJ-liposome.

Example 6

A synthetic oligonucleotide (20 bp) fluorescence-labeled with FITC (fluorescein isothiocyanate) was mixed with inactivated HVJ virus at a concentration of 1 mg/ml. After this solution was frozen and thawed twenty times, the solution was allowed to react with BMK-21 cells for 20 minutes. The fluorescence signal was observed 17 hours later. As a result, fluorescence integration was observed in the nuclei of almost 100% of the cells. From these results, it was confirmed that the method according to the present invention is also effective for introducing a synthetic nucleic acid into cells.

Example 7

After a mixed solution of GFP (Green Fluorescence Protein) gene and inactivated HVJ virus was frozen and thawed twenty times, 2 ng-5 μl of the solution was injected in a rat cerebrum. As a result, a fluorescence signal was

observed at the injection site. Moreover, the recombinant HVJ virus incorporating the GFP gene was frozen and preserved for 3 months, and thereafter injected in a rat cerebrum. Similarly, a fluorescence signal due to expression of the GFP gene was observed at the injection site.

[0028]

From the above results, it was confirmed that the method according to the present invention is certainly capable of realizing gene introduction in vivo as well. Moreover, it was also confirmed that cryopreservation of a recombinant HVJ virus is possible.

[0029]

[Effects of the Invention]

As described above in detail, the present application provides a novel gene introduction method which permits simple operation and yet provides excellent gene introduction efficiency. Moreover, the recombinant HVJ virus provided according to the present application can accept a long period of cryopreservation, so that it does not need to be prepared upon use. As a result, the operational process can be greatly simplified, and a uniform gene introduction based on mass-produced introduction vectors can be realized.

[Brief Description of Drawings]

[Figure 1]

Results of measurements of the expression (luciferase activity) level of an exogenous gene (luciferase gene), where cultured cells were transfected with a recombinant HVJ virus by freezing and thawing various numbers of times.

[Figure 2]

Results of luciferase activity measurement in the case where cultured cells were transfected, ensuring that the same number of recombinant HVJ viruses were added to the cultured cells.

[Figure 3]

Results of luciferase activity measurement in the case where the amount of an exogenous gene in a recombinant HVJ virus was variously changed.

[Figure 4]

Results of luciferase activity measurement in the case where the type of a buffer for preparation of a recombinant HVJ virus was variously changed.

[Figure 5]

Results of comparison between the gene introduction employing a conventional gene introduction vector inactivated HVJ-liposome and the method according to the present invention.

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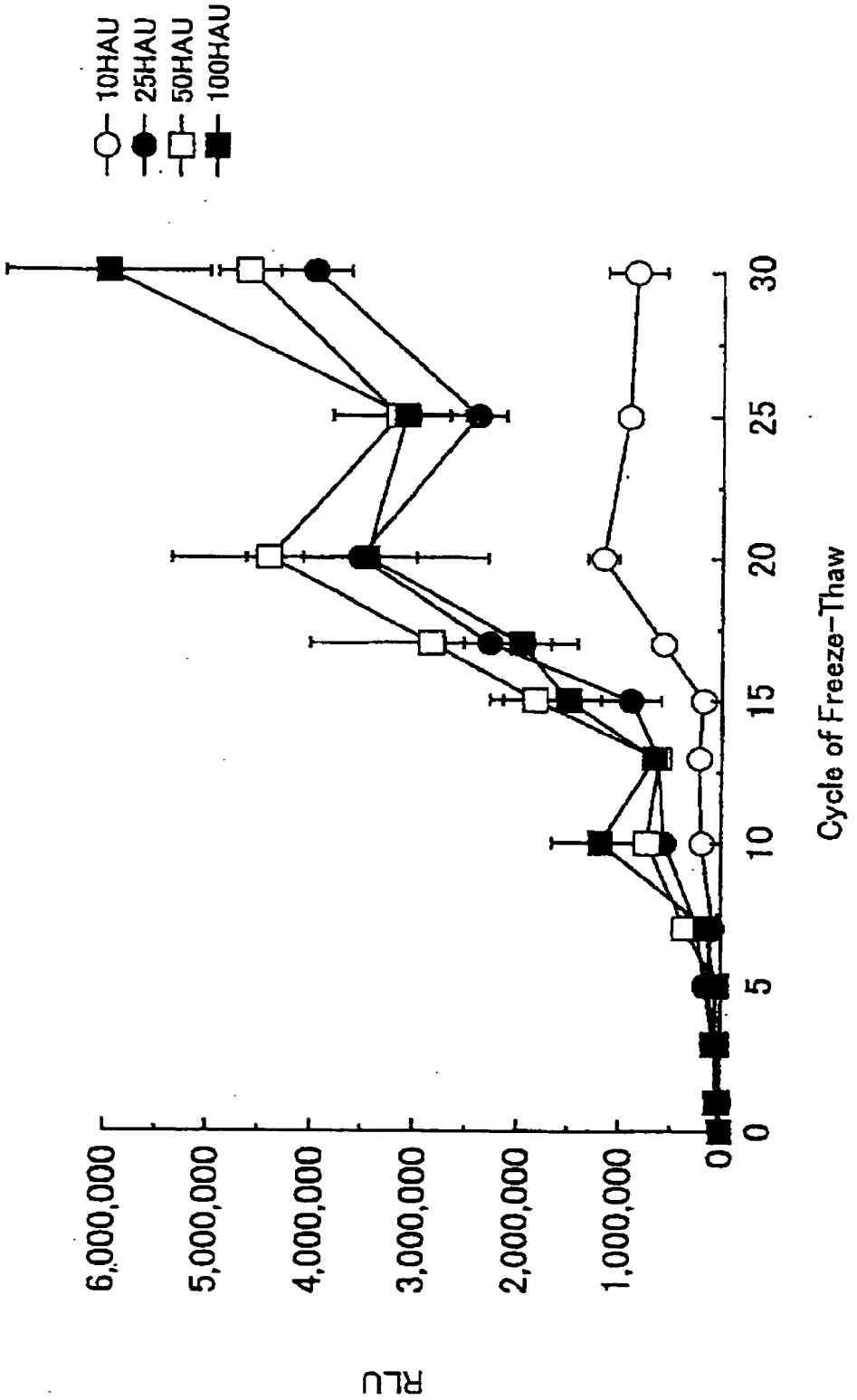
[Name of the Document] DRAWINGS

[Fig. 1]



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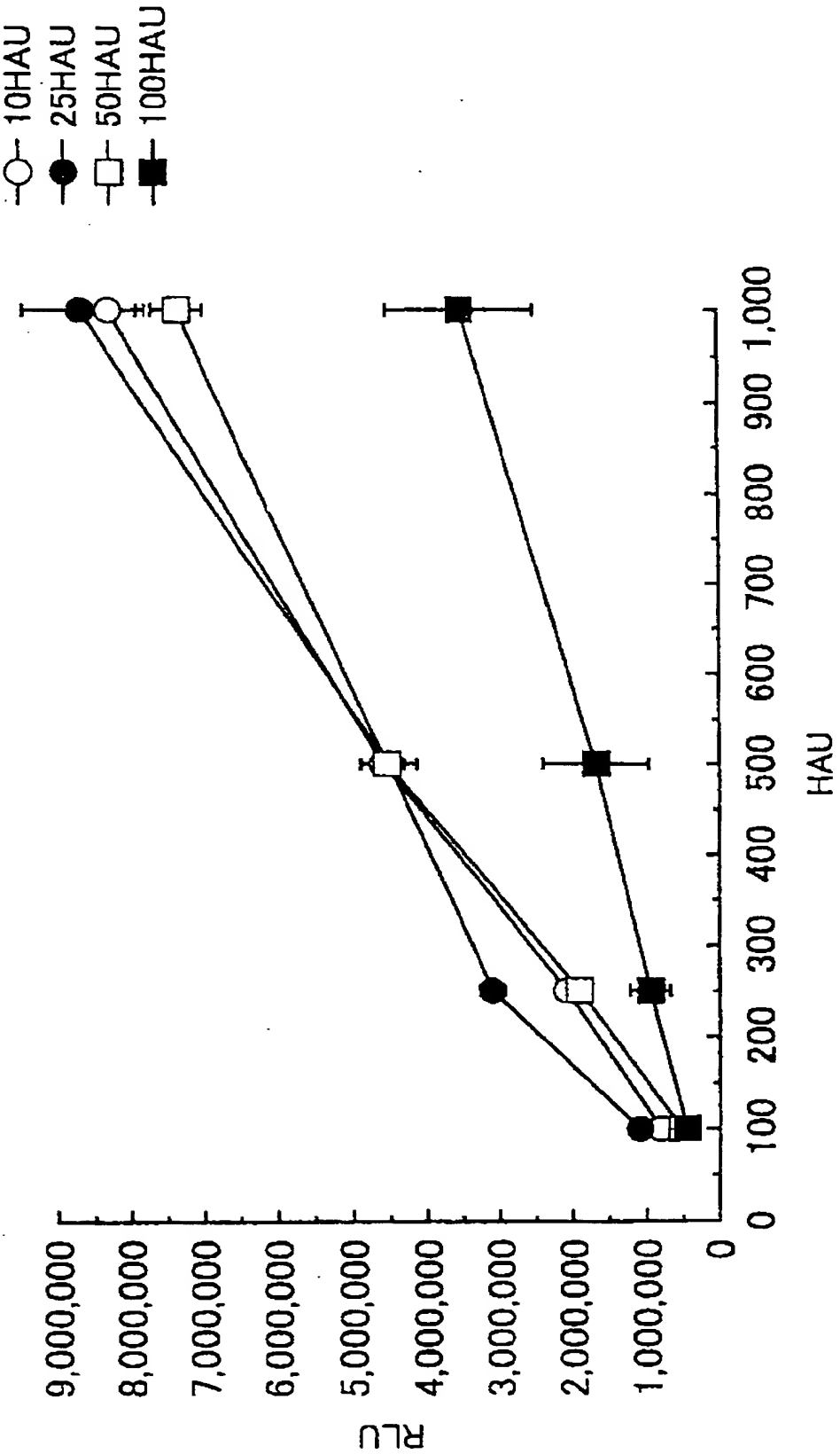
[Fig. 2]



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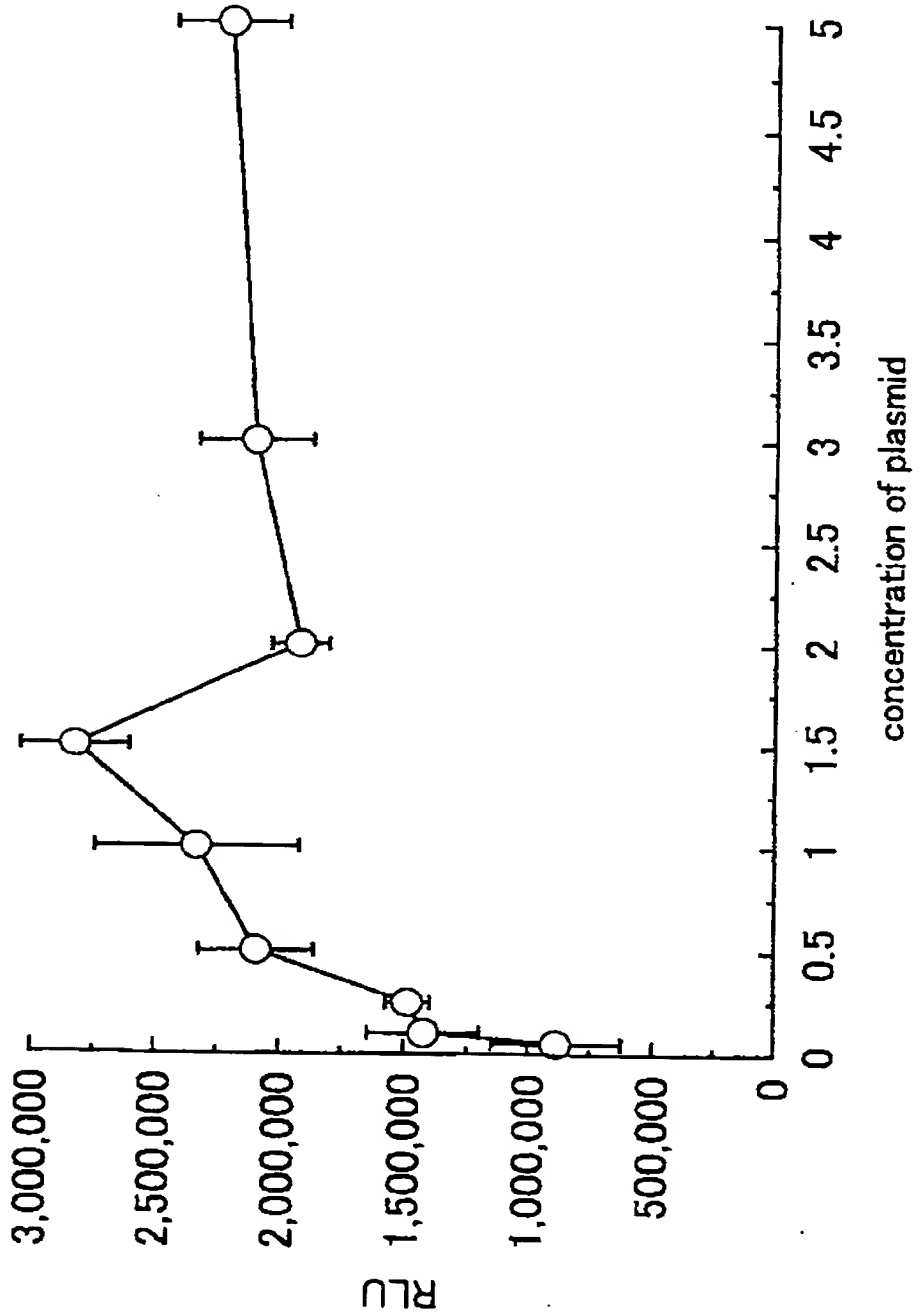
[Fig. 3]



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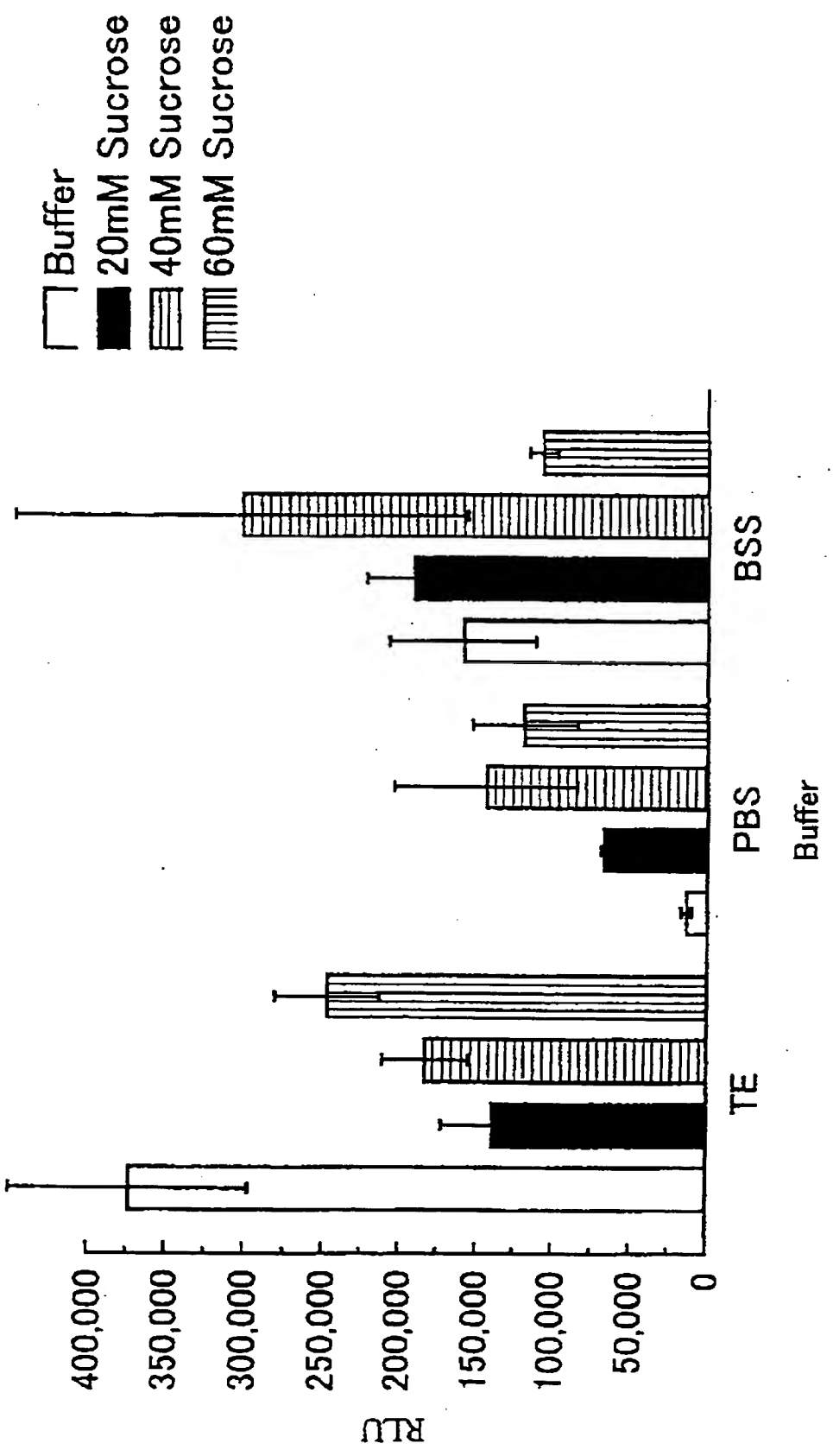
[Fig. 4]



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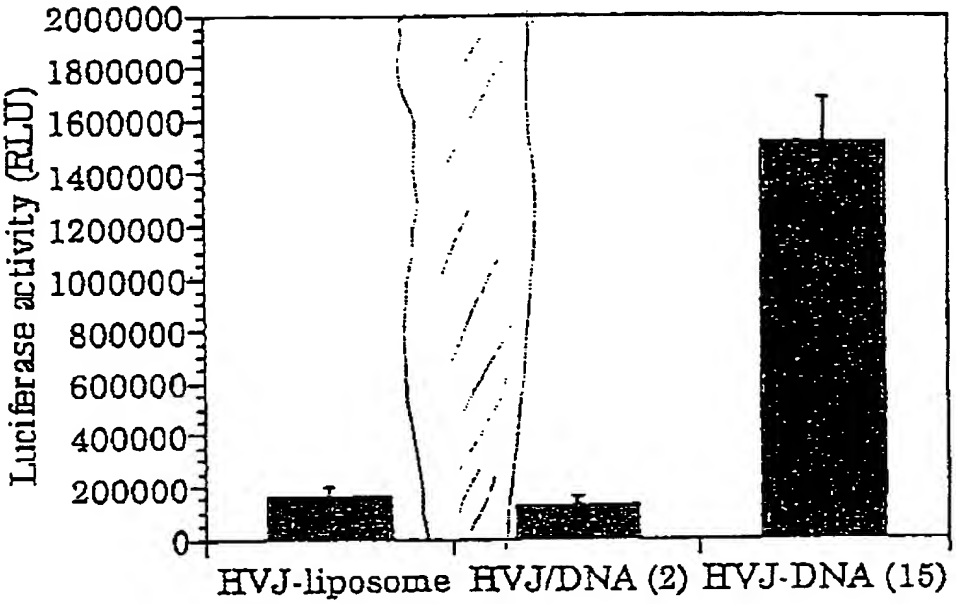
[Fig. 5]



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[Name of the Document] ABSTRACT

[Abstract]

[Problem] To provide a novel gene introduction method which permits simple operation and yet provides excellent gene introduction efficiency, and a gene introduction vector capable of long-term cryopreservation.

[Means for Solving the Problem] A method for introducing an exogenous gene, in which a mixture solution of the exogenous gene and an inactivated Sendai virus are repeatedly frozen and thawed and thereafter the virus, in which the exogenous gene is encapsulated, is fused with a cell to introduce the exogenous gene into the cell, and an inactivated Sendai virus in which an exogenous gene is encapsulated.

[Selected Figure] None